Biosynthesis of dystroglycan: processing of a precursor propeptide

Kathleen H. Holt[a], Rachelle H. Crosbie[a], David P. Venzke[a], Kevin P. Campbell[a,b,*]

[a] *Department of Physiology and Biophysics, Howard Hughes Medical Institute, University of Iowa College of Medicine, Iowa City, IA 52242, USA*
[b] *Department of Neurology, Howard Hughes Medical Institute, University of Iowa College of Medicine, Iowa City, IA 52242, USA*

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Abstract Dystroglycan is a cytoskeleton-linked extracellular matrix receptor expressed in many cell types. Dystroglycan is composed of α- and β-subunits which are encoded by a single mRNA. Using a heterologous mammalian expression system, we provide the first biochemical evidence of the α/β-dystroglycan precursor propeptide prior to enzymatic cleavage. This 160 kDa dystroglycan propeptide is processed into α- and β-dystroglycan (120 kDa and 43 kDa, respectively). We also demonstrate that the precursor propeptide is glycosylated and that blockade of asparagine-linked (*N*-linked) glycosylation did not prevent the cleavage of the dystroglycan precursor peptide. However, inhibition of *N*-linked glycosylation results in aberrant trafficking of the α- and β-dystroglycan subunits to the plasma membrane. Thus, dystroglycan is synthesized as a precursor propeptide that is post-translationally cleaved and differentially glycosylated to yield α- and β-dystroglycan.

Key words: Dystroglycan; Glycosylation; Chinese hamster ovary cell; Dystrophin–glycoprotein complex

1. Introduction

Dystroglycan is a recently characterized high-affinity receptor for several extracellular matrix components, including laminin [1–5] and perlecan [6]. The α- and β-subunits of dystroglycan are thought to arise from post-translational cleavage of a single dystroglycan propeptide. α-Dystroglycan, a peripheral membrane protein, interacts with laminin in the basal lamina and β-dystroglycan. In turn, β-dystroglycan, which crosses the membrane bilayer, associates with dystrophin and is thereby connected to the actin cytoskeleton. In addition to providing a structural linkage between the intracellular and extracellular regions of a cell, the dystroglycans are involved in agrin- and laminin-induced acetylcholine receptor clustering at the neuromuscular junctions, morphogenesis, early development and the pathogenesis of muscular dystrophies (for review, see [7]).

Dystroglycan was originally identified as a component of the dystrophin–glycoprotein complex, or DGC, which is a multisubunit group of proteins and glycoproteins resident in the sarcolemma of striated muscle. The DGC is comprised of dystrophin, a large cytoskeletal protein which binds F-actin; α- and β-dystroglycan which bind the G domain of laminin-2 and the C-terminal cysteine-rich region of dystrophin, respectively; the syntrophins; the sarcoglycan subcomplex, which is composed of four tightly associated glycoproteins named α-, β-, γ- and δ-sarcoglycan, and sarcospan (for review, see [8,9]). Functionally, the DGC stabilizes the muscle fiber sarcolemma against the considerable forces of contraction by linking the extracellular matrix and the actin cytoskeleton. The central role of the DGC in normal muscle function is underscored by discoveries that mutations in several components of the DGC give rise to distinct muscle disorders. Primary mutations in dystrophin, laminin-2 or any of sarcoglycans cause Duchenne muscular dystrophy (DMD, [10]), congenital muscular dystrophy (CMD, [11,12]) and limb-girdle muscular dystrophy (LGMD), respectively (for review, see [13]).

To date, there are no reports of dystroglycan mutations that cause muscular dystrophy in humans. However, dystroglycan structure and function are perturbed in many types of muscular dystrophy. α- And β-dystroglycan are greatly reduced from the sarcolemma in muscle from patients with DMD. In LGMD patients, α- and β-dystroglycan are localized to the sarcolemma, but α-dystroglycan is not appropriately anchored to the muscle plasma membrane [14–16]. In both of these cases, perturbation of the dystroglycan complex results in a break in the structural connection between the extracellular matrix and the cytoskeleton. Interestingly, targeted deletion of the dystroglycan gene in mice results in an early embryonic lethality due to a defect in Reichert's membrane [17]. Therefore, dystroglycan may function as an essential gene early in the development of humans prior to any assumed critical role in muscle function. Most interestingly, α-dystroglycan has been shown to act as a Schwann cell receptor for *Mycobacterium leprae* [18] and has been identified as the receptor for lymphocytic choriomeningitis virus and the lassa fever virus [19].

We recently developed a heterologous cell expression system in order to determine how primary defects in components of the DGC affect the assembly and function of this complex [20]. We first applied this in vivo methodology to examine the biosynthesis and processing of the sarcoglycan complex [20]. We reported that the molecular defect underlying LGMD is due to aberrant assembly and trafficking of the entire sarcoglycan complex [20]. We now apply this methodology to examine the importance of post-translational modifications to the processing and membrane localization of the dystroglycan complex.

α- And β-dystroglycan are tightly associated via non-covalent interactions, and together they form a direct link between the extracellular matrix protein laminin-2 and the cytoskeletal

*Corresponding author. Howard Hughes Medical Institute, University of Iowa College of Medicine, 400 Eckstein Medical Research Building, Iowa City, IA 52242, USA. Fax: (1)-319-335 6957.
E-mail: kevin-campbell@uiowa.edu

Abbreviations: CHO, Chinese hamster ovary; DGC, dystrophin–glycoprotein complex; DIC, differential interference contrast; DMD, Duchenne muscular dystrophy; LGMD, limb-girdle muscular dystrophy; *N*-linked, asparagine-linked

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protein dystrophin [1–4]. Several studies suggest that α-dystroglycan is a sialylated mucin-type glycoprotein and that this modification is necessary for laminin binding [21–23]. In addition, α-dystroglycan has several conserved sites for asparagine-linked (*N*-linked) glycosylation and glycosaminoglycan addition (for review, see [24]). The primary sequence of α-dystroglycan predicts a molecular mass of 72 kDa; however, α-dystroglycan in mammalian skeletal muscle is 156 kDa [1], 140 kDa in cardiac muscle [25] and 120 kDa in brain and peripheral nerve [26,27]. Therefore, α-dystroglycan undergoes significant post-translational modifications in a tissue-specific manner and these specialized modifications likely affect dystroglycan's function. In fact, dystroglycan is expressed in many cell types, suggesting that dystroglycan contributes to multiple cellular functions in addition to its role in skeletal muscle.

The dystroglycans are thought to be produced by a single mRNA [28]. However, there is currently no reported evidence demonstrating the presence of a dystroglycan precursor propeptide prior to cleavage. Using a heterologous mammalian expression system, we are able to provide the first evidence for an α/β-dystroglycan peptide. We also show that cleavage of the precursor propeptide into its respective α- and β-subunits can occur in the absence of *N*-linked glycosylation. Membrane localization of α- and β-dystroglycan was dependent on proper glycosylation. Furthermore, we show that this mammalian cell system is an appropriate model for examining the synthesis and post-translational processing of dystroglycan.

2. Materials and methods

2.1. cDNA expression construct

The rabbit dystroglycan expression construct was prepared by cloning an *Eco*RI fragment containing the complete cDNA for rabbit dystroglycan [28] into the *Eco*RI site of pcDNA3 (Pharmacia). Constructs were screened for directionality by restriction enzyme analysis, and sequenced by the DNA Core Facility at the University of Iowa.

2.2. Cell culture and transient transfection by electroporation

Chinese hamster ovary (CHO) cells were maintained in α-minimal Eagle's medium supplemented with nucleosides and 10% fetal bovine serum. Electroporation was performed as described previously [20]. Optimal expression was achieved using approximately 5 μg of cesium chloride gradient-purified plasmid DNA.

2.3. Antibodies

Monoclonal antibody specific to β-dystroglycan (8D5) was a generous gift of Dr. Louise V.B. Anderson (Newcastle General Hospital, Newcastle upon Tyne, UK) [29]. Sheep 005 antibody against α-dystroglycan has been described previously [30]. Polyclonal sheep antibodies specific to α-dystroglycan (Sheep 172) were raised by injection of three peptides: CAAKNHIDRVDAWVGTYFEVK, CAAKIPSDTFYDK and CAAKSWVQFNSNSQLMYGLPDSSHVGK, which correspond to amino acids 500–517, 517–526 and 548–574, respectively, of the rabbit dystroglycan sequence. Sheep 172 antibodies were affinity-purified using Immobilon-P (Millipore, Burlington, MA, USA) strips containing the bovine serum albumin (BSA)-coupled peptides CAAKNHIDRVDAWVGTYFEVK and CAAKIPSDTFYDK.

2.4. Enzymatic deglycosylation

Cell lysates (20 μg) were treated with PNGaseF as recommended by the manufacturer (Oxford Glycosystems), and analyzed by immunoblotting with the 8D5 monoclonal antibody specific to β-dystroglycan and the Sheep 172 polyclonal antibody specific to α-dystroglycan.

2.5. Tunicamycin treatment

Transfected cells were allowed to recover and adhere to culture dishes or glass cover slips for 1 h. The media were then exchanged with normal complete media or, where indicated, media supplemented with 10 μg/ml tunicamycin (Boehringer Mannheim) and incubated for an additional 30 h at 37°C in 5% CO_2.

2.6. WGA-Sepharose precipitation and immunoblotting

Whole cell detergent lysates were prepared by solubilization in lysis buffer (50 mM HEPES, pH 7.8, 300 mM NaCl, 1% NP-40 (Sigma), 1.2 mM EDTA, 5 mM $MgCl_2$, 100 mM sodium vanadate, 10 mM sodium pyrophosphate, 2 mM pepstatin, 0.5 trypsin inhibitory units of aprotinin, 1 mM phenylmethylsulfonyl fluoride and 10 μm leupeptin) and removal of insoluble material by centrifugation for 10 min in a microcentrifuge at 4°C. Clarified lysates were incubated with 20 μl of WGA-Sepharose (Pharmacia) at 4°C with rotation overnight. The WGA-Sepharose complexes were washed three times with lysis buffer and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with the monoclonal 8D5 antibody specific to β-dystroglycan or the polyclonal Sheep 172 antibody specific to α-dystroglycan. Additionally, cell culture media were collected from cell monolayers and incubated with WGA-Sepharose. The WGA-Sepharose complexes were washed three times with lysis buffer and analyzed by SDS–PAGE and immunoblotting with the monoclonal 8D5 antibody specific to β-dystroglycan or the polyclonal Sheep 172 antibody specific to α-dystroglycan. For immunoblotting, proteins were visualized using enhanced chemiluminescence (Pierce).

2.7. Immunocytochemical staining and fluorescence microscopy

Cells grown on glass cover slips in the presence or absence of tunicamycin were fixed and permeabilized in 2% paraformaldehyde/0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. The cells were blocked with 2% BSA in PBS and then incubated with monoclonal 8D5 (dilution 1:1000) or polyclonal Sheep 005 (dilution 1:500) primary antibodies. Primary antibodies were detected with fluorescently tagged secondary antibodies (Jackson Immunoresearch) used at a dilution of 1:250. Cells were imaged using a Bio-Rad MRC-1024ES laser scanning confocal microscope.

3. Results and discussion

We prepared a CMV expression vector encoding full length rabbit dystroglycan. This construct is predicted to encode an 895 amino acid precursor protein [28]. The site of cleavage on the dystroglycan propeptide has been proposed to be on the C-terminal of residue 653, such that the N-terminus of β-dystroglycan begins with Ser-654 [22,31]. However, to date there is no biochemical evidence for the existence of this precursor propeptide. β-Dystroglycan contains a transmembrane domain, while α-dystroglycan is secreted and held at the extracellular face of the membrane via non-covalent associations with the extracellular portion of β-dystroglycan.

We used a mammalian cell expression system to determine whether we could detect the presence of the dystroglycan precursor propeptide. Toward this end, we transfected CHO cells with dystroglycan cDNA encoding the precursor peptide. Thirty hours post-transfection, we analyzed cellular lysates by immunoblotting with a monoclonal antibody specific to β-dystroglycan (β-DG) (Fig. 1A) or a polyclonal antibody which recognizes α-dystroglycan (α-DG) (Fig. 1A). Both of these antibodies recognize amino acid epitopes in dystroglycan, and they are not directed against sites in the protein which contain oligosaccharide. The very broad appearance of the processed α-dystroglycan band is characteristic of the high degree of carbohydrate addition to α-dystroglycan (Fig. 1A). It is also interesting to note that the mature form of α-dystroglycan migrates at a lower molecular weight than the 156 kDa form of α-dystroglycan found in skeletal muscle; thus carbohydrate modification contributes less to the overall mass of α-dystroglycan expressed in cultured CHO cells. We also observed two bands at approximately 150 and 160 kDa

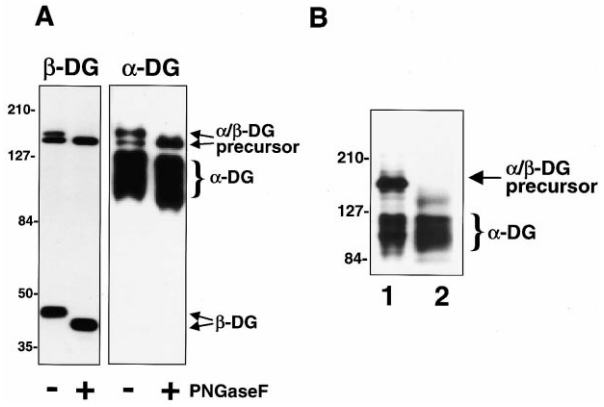

Fig. 1. *N*-linked glycosylation does not affect cleavage of the dystroglycan precursor. A: Post-translational processing of dystroglycan was examined using CHO cells transiently transfected with a plasmid encoding the α/β-dystroglycan propeptide. Cellular lysates were treated with the *N*-glycosidase, PNGaseF and subsequently examined by immunoblotting with monoclonal antibodies against β-dystroglycan (β-DG, 8D5) or polyclonal antibodies against α-dystroglycan (α-DG, Sheep 172). Control, untreated lysates (−) are shown for comparison. The dystroglycan precursor polypeptide (α/β-DG precursor) is detected by both the α-DG and β-DG antibodies. Molecular size standards are indicated on the left ($\times 10^3$ Da). B: The mature form of α-dystroglycan is secreted into the cell culture media. Detergent extracts from CHO cells transiently transfected with plasmid encoding the α/β-dystroglycan propeptide were incubated with WGA-Sepharose to precipitate dystroglycan (lane 1). At the same time, WGA-Sepharose was used to enrich dystroglycan that may have been released from the CHO cells into the cell culture media (lane 2). Proteins bound to the WGA-Sepharose were analyzed by Western blotting with antibodies to α-dystroglycan. Mature α-dystroglycan was secreted into the culture media (lane 2), however, the α/β-dystroglycan precursor remained in the cellular lysate (lane 1). Molecular size standards are indicated on the left ($\times 10^3$ Da).

that were recognized by α- and β-dystroglycan antibodies. To evaluate the extent of *N*-linked glycosylation of both the mature and propeptide forms of dystroglycan, cell extracts were subjected to enzymatic deglycosylation using PNGaseF. As shown in Fig. 1A, we observed an increase in mobility in SDS gels in both β-dystroglycan and α-dystroglycan following treatment with PNGaseF. This result confirms that α- and β-dystroglycan are recipients of *N*-linked glycosylation in this expression system. The broad appearance of α-dystroglycan remains after PNGaseF treatment, suggesting that *O*-linked glycosylation is contributing to the smeared staining pattern of this band. Interestingly, the precursor peptide appeared as a doublet in untreated samples, and the upper band (160 kDa) shifted down to the size of the lower band (150 kDa) after deglycosylation (Fig. 1A). This result indicates that the precursor peptide acquires *N*-linked oligosaccharides prior to the cleavage event. The data demonstrate that the 160 kDa precursor propeptide is processed into a 43 kDa protein (β-dystroglycan) and a 120 kDa protein (α-dystroglycan). The fact that both the monoclonal β-dystroglycan and the polyclonal α-dystroglycan antibodies recognize the precursor peptide as well as their respective cleaved subunits verifies these findings.

Recent reports have demonstrated that RT4 schwannoma [32] and bovine aortic endothelial [33] cells secrete a soluble form of α-dystroglycan into the culture medium. Neither of these reports provided data which would explain why α-dystroglycan was released into the medium, rather than being anchored securely at the extracellular face of the plasma membrane via associations with β-dystroglycan. The BIO14.6 hamster is a dystrophic animal model for LGMD, with a defined mutation in the δ-sarcoglycan gene that results in the loss of the entire sarcoglycan complex at the sarcolemma [34]. Recent studies of the BIO14.6 hamster from our laboratory and others demonstrate that the sarcoglycan complex is required for stable association of α-dystroglycan at the membrane [14–

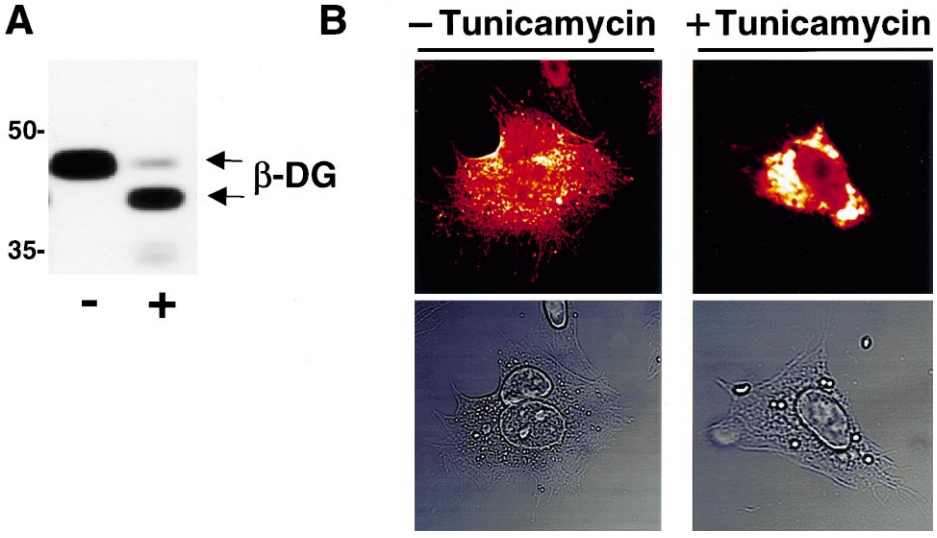

Fig. 2. Tunicamycin treatment inhibits trafficking of β-dystroglycan to the surface plasma membrane. (A) Prevention of *N*-linked glycosylation does not block cleavage of the dystroglycan precursor. CHO cells were transfected with the α/β-dystroglycan construct and grown in the absence (−) or presence (+) of tunicamycin, as detailed in Section 2. Whole cell lysates were analyzed by immunoblotting using antibodies directed against β-dystroglycan (8D5). The presence of β-dystroglycan in tunicamycin-treated cells indicates that *N*-linked glycosylation is not necessary for cleavage of the α/β-dystroglycan precursor. Tunicamycin efficiently blocks *N*-linked glycosylation, as shown by the shift in molecular mass of β-dystroglycan. (B) CHO cells expressing the α/β-dystroglycan construct were grown in the presence (+) or absence (−) of tunicamycin. Cells were fixed with paraformaldehyde and analyzed by immunofluorescence for the expression of β-dystroglycan (upper panels) using antibodies against β-dystroglycan (8D5). The cells were viewed in the same focal planes using DIC in order to image the cell borders (lower panels).

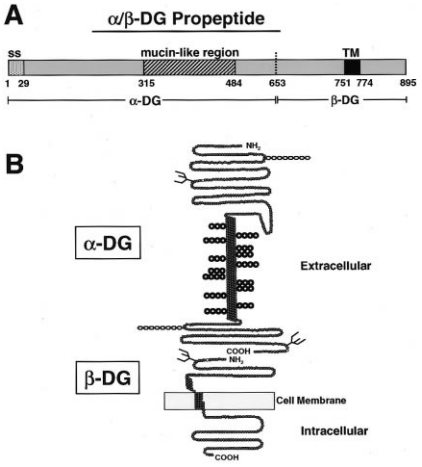

Fig. 3. Biosynthetic intermediates of dystroglycan. A: Schematic depiction of the domain organization of the α/β-dystroglycan propeptide. The dystroglycan propeptide has an N-terminal signal sequence (ss), a mucin-like region, and a single transmembrane domain (TM). Amino acid numbers are given below the diagram. The α/β-dystroglycan propeptide consists of 895 amino acid residues and is predicted to have a core molecular mass of 97 kDa. Proteolytic cleavage of the dystroglycan propeptide occurs after residue 653 (dashed line) to produce α- and β-dystroglycan subunits. α-Dystroglycan undergoes significant post-translational modifications and the molecular mass of this protein can vary between 120 and 156 kDa, depending on the tissue from which it is isolated. B: Representative structure of the mature α- and β-dystroglycan polypeptides. α-Dystroglycan is depicted as a dumb-bell-shaped structure, with N- and C-terminal globular domains separated by an extended mucin-like region. Extensive *O*-linked glycosylation (○) within this region likely mediates interactions with laminin in the extracellular matrix. Potential sites for attachment of glycosaminoglycan chains are indicated with hexagons and sites for *N*-linked carbohydrate modification are represented with branches. α-Dystroglycan non-covalently interacts with β-dystroglycan, which resides within the cellular membrane. In turn, the C-terminus of β-dystroglycan is associated with dystrophin, thereby completing the structural link between the extracellular matrix and the intracellular cytoskeleton.

16]. Therefore, we speculate that α-dystroglycan may not be held securely at the membrane in cultured non-muscle cells which lack the sarcoglycan complex.

We looked for the presence of soluble α-dystroglycan in the conditioned medium of our CHO cells expressing dystroglycan. WGA-Sepharose precipitates from whole cell detergent extract (Fig. 1B, lane 1) and conditioned cell culture medium (Fig. 1B, lane 2) from cells expressing dystroglycan were analyzed by immunoblotting with the polyclonal antibody against α-dystroglycan (α-DG). Both the precursor peptide and the mature form of α-dystroglycan were readily precipitated by the WGA-Sepharose from the cell lysate. In contrast, only the fully processed form of α-dystroglycan was detected in the culture medium. This result is not surprising, because the dystroglycan precursor peptide contains the transmembrane domain, and therefore we would not expect it to be competent for secretion.

To further evaluate the contribution of *N*-linked oligosaccharide addition to the maturation and trafficking of dystroglycan, we performed experiments using tunicamycin. Tunicamycin is a glycosylation inhibitor that blocks the transfer of dolichol pyrophosphate precursor to asparagine in the first step of *N*-linked oligosaccharide addition. In Fig. 2A, cells expressing dystroglycan were left untreated (−) or treated (+) with tunicamycin. Equal quantities of protein were loaded onto SDS–polyacrylamide gels and then analyzed by immunoblot using antibodies against β-dystroglycan. Tunicamycin treatment resulted in a nearly complete block in *N*-linked glycosylation as evidenced by the smaller molecular weight of β-dystroglycan (Fig. 2A). Interestingly, cleavage of the precursor peptide can proceed in the absence of *N*-linked glycosylation, as evidenced by the presence of the smaller non-glycosylated form of β-dystroglycan, which is detected by the β-dystroglycan monoclonal antibody (Fig. 2A).

Finally, to confirm that *N*-linked oligosaccharide addition was essential for successful biosynthesis and trafficking of α- and β-dystroglycan, we examined the cellular localization of these proteins by confocal microscopy. CHO cells expressing dystroglycan were grown on glass cover slips in the absence or presence of tunicamycin. Using immunofluorescence with the β-dystroglycan antibody, we demonstrate that β-dystroglycan was localized to the peripheral cell membranes when the cells were grown in the absence of tunicamycin (Fig. 2B). The cells grown in the presence of tunicamycin exhibited a dramatic relocalization of β-dystroglycan to intracellular perinuclear structures (Fig. 2B). α-Dystroglycan localization was also cytosolic by the treatment with tunicamycin (data not shown). The cells were also imaged using differential interference contrast (DIC) optics to clearly delineate the cell borders (Fig. 2B). Tunicamycin treatment did not affect the gross appearance, cell growth or viability of the transfected CHO cells. These results are consistent with the idea that *N*-linked glycosylation is a requisite step in the sequence of events for normal processing and cell surface localization of the α- and β-dystroglycan subunits.

We have employed a heterologous cell system to study the biosynthesis and maturation of the α- and β-dystroglycan propeptide. First, we provide evidence for the existence of the uncleaved α- and β-dystroglycan propeptide and we have summarized these findings in Fig. 3. We show that this 160 kDa precursor is processed into α- and β-dystroglycan (120 kDa and 43 kDa, respectively). The precursor polypeptide has never been observed in native tissue, likely because it is rapidly cleaved into α- and β-dystroglycan. Our overexpression of dystroglycan in cultured CHO cells has enabled us to 'catch' the glycosylated and non-glycosylated forms of the dystroglycan precursor protein prior to cleavage. We have also addressed what role *N*-linked oligosaccharide addition plays in the sequence of events that are required for successful dystroglycan maturation. We provide convincing evidence that *N*-linked glycosylation is not an absolute prerequisite for cleavage, because the precursor peptide is readily cleaved when *N*-linked glycosylation is blocked by tunicamycin. On the other hand, *N*-linked glycosylation is required for the cell surface localization of both α- and β-dystroglycan. In the future, we hope to use this model system to study the binding potential of α-dystroglycan to various extracellular matrix components, as well as the association of β-dystroglycan with the cytoskeletal proteins, dystrophin and utrophin. We also expect such studies to reveal how specific post-translational modifications of α- and β-dystroglycan modulate protein–protein interactions.

In recent years, genetic approaches have provided enormous advances in our understanding of the central role played by the DGC in muscle physiology. We now understand that mutations in various components of the DGC can independ-

ently, and to varying degrees, result in structural and functional deficits in the DGC, which inevitably lead to muscle fiber damage and necrosis. Dystroglycan is notably absent from the list of DGC members which have been genetically linked to forms of human muscular dystrophy. Recently, a dystroglycan knock out mouse has been reported to suffer an early embryonic lethal phenotype, suggesting that by way of analogy, mutations in dystroglycan may be deleterious in humans [17]. The role of dystroglycan in normal muscle function must await the generation of a conditional allele of dystroglycan, which can be turned off in muscle under controlled conditions.

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